Expression and Characterization of Transforming Growth Factor α Precursor Protein in Transfected Mammalian Cells
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Received 10 November 1986/Accepted 26 January 1987

Analysis of a cDNA clone derived from retrovirus-transformed rat fibroblasts has recently suggested that the mature 50-amino-acid form of transforming growth factor α (TGФα) is derived from a 159-amino-acid transmembrane precursor by proteolytic cleavage. To understand the processing of the TGФα precursor molecule in more detail, we have expressed this protein in baby hamster kidney (BHK) fibroblasts under control of the metal-ion-inducible metallothionein promoter and characterized the expressed precursor with site-specific antipeptide antibodies. One of the BHK transfectants, termed 5:2, expressed the TGФα mRNA in a cadmium- and zinc-inducible manner. The TGФα precursor protein was detected by immunoprecipitation analysis of radiolabeled cell cultures. In the induced 5:2 cells, a polypeptide of Mr 13,000 to 17,000 was readily identified by peptide antisera made to three different regions of the TGФα precursor protein. No such protein species were observed in BHK cells treated with cadmium and zinc or in uninduced 5:2 cells. However, two cell lines known to produce TGФα naturally, Leydig testicular tumor cells and Snyder-Thelain feline sarcoma virus-transformed Fisher rat embryo fibroblasts, possessed detectable levels of immunologically related Mr 13,000 to 17,000 proteins. Cell fractionation studies indicate that the Mr 13,000 to 17,000 species expressed in induced 5:2 cells is membrane associated, consistent with predictions based on the cDNA sequence of the TGФα precursor. Media conditioned by induced 5:2 cells contained epidermal growth factor receptor-competing activity, which, upon size fractionation, was similar in size to the mature processed form of TGФα. These data show that nontransformed BHK cells possess the ability to process the TGФα precursor molecule into its native form.

Transforming growth factor α (TGФα) is a small polypeptide, of approximately 50 amino acids, which appears to be structurally and functionally related to epidermal growth factor (EGF) (15, 16). These polypeptides show significant sequence homology and appear to have evolved by gene duplication from a common ancestral molecule (15). TGФα and EGF compete for binding to the same cell surface receptor, the EGF receptor, and activate the associated tyrosine kinase activity of the receptor molecule (17, 18). Although these growth factors are potent mitogens in tissue culture, recent reports have suggested that TGФα may have different biological properties from those of EGF. TGФα has been reported to be more potent at promoting calcium release from rat fetal long bones (6, 7) and as an angiogenesis factor (20).

TGФα, initially identified in the medium of retrovirus-transformed rodent cells (1, 26, 27; for reviews, see reference 24 and D. R. Twardzik and J. R. Ranchalis, UCLA Symp. Bone Cartilage, in press), is expressed in a variety of human tumors and tumor cell cultures (16, 23) and is thought to play a role in the malignancy process. Analysis of the cloned TGФα cDNA has revealed some interesting features regarding its expression. As is true of several other growth factors, TGФα appears to be processed from a larger precursor molecule. In the rat and the human, the precursors are 159 and 160 amino acids, respectively, and each contains a typical leader sequence and an extremely hydrophobic domain resembling a transmembrane region (2, 11). This led to the prediction that the mature 50-amino-acid form of TGФα is derived by proteolytic release from a membrane-bound precursor molecule. Of particular interest is the finding that proteolysis occurs at unique Ala-Val-Val sequences and is most probably mediated by a protease having elastase-type specificity (11; D. C. Lee, T. M. Rose, N. R. Webb, D. R. Twardzik, J. R. Ranchalis, H. Marquardt, and G. J. Todaro, Perspect. Med. [R. Soc. Lond. Symp.], in press). The fact that an unusual protease is involved in the processing of the precursor raises the possibility that proteolysis is a regulatory event in the generation of the mature growth factor. Moreover, the marked sequence conservation of the entire precursor that is evident in a comparison of the human and rat cDNAs may suggest that the precursor has functions distinct from those of the mature growth factor.

To understand the structure and processing of the TGФα precursor in more detail, we have constructed an expression vector that places a portion of the rat TGФα cDNA which encodes the complete TGФα precursor molecule under the control of the inducible mouse metallothionein promoter. This construct has been introduced into baby hamster kidney (BHK) fibroblasts, and the precursor protein produced in these cells has been characterized with site-specific antipeptide antibodies. Our results demonstrate that the TGФα precursor is a membrane-associated polypeptide with Mr 13,000 to 17,000 that is proteolytically processed in BHK

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Vol. 7, No. 5
MOLECULAR AND CELLULAR BIOLOGY, May 1987, p. 1585-1591
0270-7306/87/051585-07$02.00/0
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fibroblasts to release a mature M, 6,000 TGfα form into the culture medium.

**MATERIALS AND METHODS**

**Cell culture and radioactive labeling.** Fisher rat embryo fibroblast (FRE) cells and their Snyder-Theilen feline sarcoma virus-transformed counterparts were from previously described clones (27). Leydig testicular tumor cells were generously provided by Greg Mundy. BHK fibroblasts were provided by Richard Palmiter. Leydig tumor cells were grown in RPMI 1640 supplemented with 15% fetal bovine serum. All other cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Confluent cultures of cells were labeled with [35S]cysteine (>1,000 Ci/mmol; New England Nuclear Corp.) in cysteine-free Dulbecco modified Eagle medium containing 5% diazotized fetal bovine serum. FRE, ST-FeSV-transformed FRE, and Leydig tumor cells were labeled at 500 µCi/ml. For experiments involving BHK cells or the 5:2 transfected, cells were induced with 2 µM cadmium sulfate and 100 µM zinc chloride prior to being labeled. Following a 6- to 10-h induction period, cells received cysteine-free DME supplemented with 5% diazotized fetal bovine serum, 200 µCi of [35S]cysteine per ml, and an equivalent amount of metal ion inducer.

**Construction of the metallothionein-rat TGfα plasmid.** The TGfα expression vector, was constructed by using plasmid EV142 (13), which contains the mouse metallothionein 1 promoter fused to the 3′ untranslated region of the human growth hormone gene (kindly provided by Richard Palmiter). The mouse metallothionein 1 sequence is contained in an 800-base-pair (bp) fragment which extends from the 5′ flanking sequence through the promoter and includes the first 64 bp of exon 1. This fragment is fused by a unique BglII site to a 625-bp human growth hormone fragment which contains the 3′ untranslated region of exon 5, the polyadenylation signal, and 3′ flanking sequences. These fragments are contained in pBKB322, a high-copy-number derivative of pBR322 which contains an XhoI linker at about position 3000. To generate pEV2, a 700-bp Smal fragment which includes the entire rat TGfα coding region was inserted into the unique BglII site. Clones with the proper orientation were selected, and DNA was isolated for transfection.

**Transfection of BHK cells.** Subconfluent BHK cells grown in 100-mm dishes containing 10 ml of complete medium (Dulbecco modified Eagle medium with 10% fetal bovine serum) were transfected with 20 µg of pEV2 as a calcium phosphate precipitate (28) in the presence of 1 mM chloroquine. After the cells had been incubated for 5 h at 37°C, the medium was removed and replaced with 10 ml of fresh medium. After an additional 24 h in culture, Geneticin (G418; GIBCO Laboratories) was added to the medium at 500 µg/ml. Antibiotic-resistant clones were visible after 1 week; these clones were isolated and tested for their expression of TGfα mRNA by a solution hybridization assay (D. C. Lee, K. J. Ibbotson, S. M. D’Souza, C. Reasner, G. Carpenter, D. R. Twardzik, G. J. Todaro, and G. R. Mundy, J. Clin. Invest., in press).

**RNA isolation and Northern blot analysis.** RNA was isolated by homogenization of tissue or cells in 4 M guanidine thiocyanate and sedimentation through 5.7 M CsCl (5). Northern blots were performed essentially as described previously (10) with poly(A)⁺ RNA selected by chromatography on oligo(dT)-cellulose.

**Production of antipeptide antibodies.** Peptides were synthesized by solid-phase techniques on a Beckman automated instrument (4) and purified by preparative high-pressure liquid chromatography. The composition of the peptides was confirmed by amino acid analysis. Purified peptides were coupled to bovine γ-globulin through the cysteine residue and used to immunize New Zealand White rabbits (3). Immunization schedules were as described previously (3).

**Immunoprecipitation analysis.** Radiolabeled cell cultures were lysed in detergent-containing buffer (20 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.5% sodium deoxycholate, 1.0% Nonidet P-40, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 200 kallikrein inhibitory units of aprotinin [Sigma Chemical Co.] per ml) and clarified by centrifugation at 100,000 × g for 30 min at 4°C. A 100-µl portion of lysate equivalent to 5 × 10⁵ to 1 × 10⁶ cells was precleared with 10 µg of Staphylococcus aureus and then incubated with 5 µl of serum at 0°C for 1 h. Immunocomplexes were collected on S. aureus and washed as described previously (3). Peptide-blocking experiments were performed with purified peptide. Immunoprecipitated samples were lysed with sodium dodecyl sulfate (SDS)-containing buffer and electrophoresed on 15% polyacrylamide or 12 to 20% gradient polyacrylamide gels (9). Radiolabeled proteins were detected by fluorography with En³Hance (New England Nuclear). \[ "\text{Radioimmunoassay.} \text{The binding of 125I-labeled EGF to its receptor was measured on monolayers of formalin-fixed A431 cells as previously described (1). Concentrations of TGfα are expressed as nanogram equivalents of EGF, i.e., the amount required to produce an inhibition of 125I-labeled EGF binding equivalent to that produced by a known amount of EGF.}\]

**Membrane fractionation.** Radiolabeled cell cultures were collected by scraping. Cells were swollen in hypotonic lysis buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; pH 6.9], 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 200 kallikrein inhibitory units of aprotinin per ml) at 2 × 10⁷ cells per ml for 20 min at 4°C and Dounce homogenized. Nuclei and unbroken cells were removed by centrifugation at 2,000 × g for 15 min at 4°C, and the supernatant was further fractionated into pelleting (P100) and supernatant (S100) fractions by centrifugation at 100,000 × g. The P100 fraction was separated on discontinuous sucrose gradients essentially as described previously (14). For immunoprecipitation analysis, samples were adjusted to 1× in detergent lysis buffer.

**RESULTS**

**Expression of rat TGfα in BHK cells.** To characterize the TGfα precursor, we prepared an expression vector (pEV2; Fig. 1A) that places the rat TGfα cDNA under the control of the inducible mouse metallothionein 1 promoter. Induced transcription of this vector would yield a hybrid mRNA of 1 kilobase pair (kb) containing 700 base pairs (bp) of TGfα cDNA, 64 bp of metallothionein gene, and 240 bp of the human growth hormone segment. The last of these was present to provide a 3′ noncoding sequence and polyadenylation signal. pEV2 was cotransfected into BHK cells along with the selectable neomycin resistance marker, and cells resistant to G418 were isolated. Expression of TGfα mRNA in transfected BHK cells was determined by solution hybridization as described previously (4). A transfected cell line (termed 5:2) was shown to express the hybrid TGfα mRNA at high levels following induction with cadmium and zinc and was used for further characterization.
A Northern blot analysis of poly(A)+ RNA isolated from 5:2 cells is shown in Fig. 1B. Equivalent amounts of poly(A)+ RNA from rat liver and Leydig testicular tumor cells, a cell line known to produce TGFα protein, were included for comparison. Induced 5:2 cells contained a large amount of 1-kb mRNA that hybridized to the TGFα cDNA probe. The levels of this hybrid 5:2 mRNA appeared to be similar to, if not greater than, the levels observed for the natural 4.5-kb mRNA found in Leydig cells. In contrast, uninduced 5:2 cells contained nondetectable amounts of TGFα mRNA. Using the solution hybridization assay, we estimated that the hybrid TGFα mRNA was induced at least 20- to 50-fold in 5:2 cells after a 10- to 12-h exposure to cadmium and zinc.

Immunoprecipitation of the TGFα precursor polypeptide with site-specific antipeptide antibodies. Site-specific antibodies directed toward peptide sequences within the predicted rat TGFα precursor molecule (11) were generated in rabbits by using synthetic peptides as immunogens. Figure 2A shows the peptide sequences which were used, as well as their relative locations within the TGFα precursor polypeptide. The TGFα antibodies were directed toward epitopes present within the carboxy-terminal region of the precursor molecule and are precursor specific.

Antiserum were tested for their ability to specifically immunoprecipitate induced proteins from [35S]cysteine-labeled cell cultures. The results of the immunoprecipitation analysis are shown in Fig. 2B. In the cadmium- and zinc-induced 5:2 cells (lanes 5:2), prominently labeled proteins of Mr 13,000 to 17,000 were readily identified by each of the peptide antiserum. This species was not identified by normal rabbit serum and was not immunoprecipitated from control BHK cells exposed to similar levels of cadmium and zinc (lanes BHK) or in uninduced 5:2 cells (lanes 5:2). Furthermore, incubation of peptide antiserum with synthetic peptide immunogen prior to addition of radiolabeled cell lysate completely abolished detection of this protein species (lane 5:2+PEP). Two additional TGFα antiserum, one directed against a 17-amino-acid synthetic peptide constituting the carboxy-terminus of the fully processed TGFα (12) and one generated against the synthetic 50-amino-acid TGFα molecule, identified proteins which comigrated with the Mr 13,000 to 17,000 species recognized by the precursor-specific antibodies (data not shown).

SDS-polyacrylamide gel electrophoresis of immunoprecipitates from [35S]cysteine-labeled FRE, ST-FeSV-transformed FRE, and Leydig testicular tumor cells are shown in Fig. 3. Both transformed cell types, known to produce TGFα naturally, exhibited an immunoreactive protein species which appeared to comigrate with the species observed in 5:2 cells. This Mr 13,000 to 17,000 protein was identified by two different TGFα antiserum and in a peptide-specific manner. Furthermore, this protein was not detected in the nontransformed FRE cells. These results provide strong evidence that the Mr 13,000 to 17,000 species identified in the BHK transfectants and in the ST-FeSV-transformed FRE and Leydig cell lines is the TGFα precursor and are consistent with the size (Mr 16,970) of the precursor molecule predicted on the basis of the cloned cDNA sequence (11). Interestingly, the Mr 13,000 to 17,000 TGFα precursor polypeptide appeared more abundant in the transfectant BHK cell line than in either ST-FeSV-transformed FRE or Leydig cells.

In addition to the Mr 13,000 to 17,000 protein species identified in ST-FeSV-transformed FRE and Leydig tumor cells, a protein of Mr 25,000 was detected in the immunoprecipitates. This protein was present in both transformed cell types, was absent from nontransformed FRE cells, and
Symbols: \[ (B) \text{ Gel} \] the-\[zw\]insr \[511UWIIIt; \] amide gradient forms of bioactive are used metal blocked cells treated with larger species markers immunoprecipitation analysis of to nologically \[25,000 \] BHK cells. The TGF\[a\] indicated antibodies. Confluent cultures of cells were labeled for 2 h with 0.5 mCi of \[^{35}\text{S}]\text{cysteine} per ml in cysteine-free media. Immunoprecipitates from equivalent amounts of \[^{35}\text{S}]\text{cysteine}-labeled cells were resolved on SDS-polyacrylamide gel electrophoresis and analyzed for its ability to compete with iodinated EGF for binding to the EGF receptor. As controls, we included the parental BHK cells exposed to cadmium and zinc, as well as the uninduced 5:2 cells. ST-FeSV-transformed FRE cells known to produce high levels of mature, fully processed TGF\[a\] were used for comparison. Figure 4A shows the results obtained. ST-FeSV-transformed FRE cells produced by far the greatest amount of EGF-competing activity into culture supernatants, approaching nearly 6 ng equivalents of EGF-competing activity per ml of conditioned culture medium. Induced 5:2 cells also contained competing activity, albeit at lower levels (ca. 0.8 ng equivalents per ml of conditioned medium). Supernatants from uninduced 5:2 cells and the parental BHK cell line treated with cadmium and zinc produced nondetectable levels of EGF-competing activity.

To examine the nature of the TGF\[a\] molecule secreted by the induced 5:2 cells, conditioned medium was fractionated over a high-pressure liquid chromatography gel permeation column and fractions were assayed for EGF-competing activity (Fig. 4B). The EGF-competing activity from induced 5:2 cells was detected as a \[M \] 6,000 protein species which appeared to coelute with the fully processed TGF\[a\] form found in the supernatant of ST-FeSV-transformed FRE cells. No larger forms of TGF\[a\] were detected, indicating that processing of the precursor was complete in these cells.

Detection of processing intermediates. Pulse-chase analysis was used to identify processing intermediates of TGF\[a\]. Induced 5:2 cells were pulse-labeled in cysteine-free media for 15 min with \[^{35}\text{S}]\text{cysteine} and chased with complete media for various times. Detergent lysates were prepared, and the TGF\[a\] precursor was immunoprecipitated with anti-TGF\[a\]. The TGF\[a\] precursor was readily identified in the induced 5:2 cells by using a 15-min labeling period. At time zero, the TGF\[a\] precursor behaved as a polypeptide migrating on SDS-polyacrylamide gels with \[M \] 14,000 to 18,000. After a 20-min chase, an increase in the size of the TGF\[a\] precursor polypeptide from \[M \] 14,000 to 18,000 to \[M \] 15,000 to 19,000 was readily apparent. Longer chase times revealed the dramatic decrease in size of the \[M \] 15,000 to 19,000

FIG. 2. (A) Line diagram of the rat TGF\[a\] precursor protein, showing the location and sequences of synthetic peptide immunogens. Symbols: \[ , \] functionally important regions of the precursor. (B) Gel electrophoresis of immunoprecipitates prepared from antipeptide antibodies. Immunoprecipitates prepared from \[^{35}\text{S}]\text{cysteine}-labeled cells were fractionated on 12 to 20% polyacrylamide gradient gels and fluorographed. Cells were induced with metal ions for 8 h and labeled for 12 h with \[^{35}\text{S}]\text{cysteine}. Antisera used are indicated at the top of the figure. Lanes: BHK, BHK cells treated with cadmium and zinc; 5:2, uninduced 5:2 cells; 5:2i, 5:2 cells treated with cadmium and zinc; 5:2i + PEP, antibody was blocked with 0.2 \(\mu\)g of synthetic peptide immunogen prior to immunoprecipitation analysis of induced 5:2 cells. Molecular mass markers are indicated on the right.

appeared to be identified in a peptide-specific manner. This larger species may be related to higher-molecular-weight forms of bioactive TGF\[a\] secreted by some tumor cell lines (12). Alternatively, this polypeptide may represent an immunologically distinct form associated with the \[M \] 13,000 to 17,000 TGF\[a\] precursor during immunoprecipitation. The \[M \] 25,000 form was not detected in the transfected BHK cells.

The TGF\[a\] precursor protein is proteolytically processed in BHK cells. To determine whether the TGF\[a\] precursor protein was secreted or processed in the transfected BHK cells, conditioned medium was collected from these cells and analyzed for its ability to compete with iodinated EGF for binding to the EGF receptor. As controls, we included the parental BHK cells exposed to cadmium and zinc, as well as the uninduced 5:2 cells. ST-FeSV-transformed FRE cells

FIG. 3. Immunoprecipitation of TGF\[a\] from Leydig testicular tumor and ST-FeSV-transformed FRE cells with precursor-specific antipeptide antibodies. Confluent cultures of cells were labeled for 2 h with 0.5 mCi of \[^{35}\text{S}]\text{cysteine} per ml in cysteine-free media. Immunoprecipitates from equivalent amounts of \[^{35}\text{S}]\text{cysteine}-labeled proteins were prepared and analyzed by SDS-polyacrylamide gel electrophoresis on 15% polyacrylamide gels. Molecular mass markers (kilodaltons) are shown on the left. *PGE-treated lanes which were preincubated with synthetic peptide prior to immunoprecipitation. Only the bottom two-thirds of the gel is shown.
species to one of $M_r$ 13,000 to 17,000. After a 160-min chase, the detected TGFα precursor species comigrated with the precursor immunoprecipitated from induced 5:2 cells labeled for 8 h (control lane), suggesting that complete processing had occurred over this 160-min chase period. The half-life of the precursor identified with anti-TGFα 137-159 was approximately 2 h. We cannot rule out the possibility that this apparent half-life is due to an early cleavage event involving the carboxy-terminal epitopes used for immunoidentification.

The TGFα precursor protein is membrane associated. The membrane localization of the TGFα precursor was determined by cell fractionation and differential centrifugation.

Radiolabeled 5:2 cells treated with cadmium and zinc were broken, and the microsomal fraction and soluble constituents were separated by ultracentrifugation. Both the S100 and P100 fractions from a 100,000 $\times$ g centrifugation were tested by immunoprecipitation. The results are shown in Fig.

FIG. 4. (A) EGF-competing activity of conditioned medium from tissue culture cells. Conditioned medium prepared as described in Materials and Methods was tested for its ability to compete with iodinated EGF for binding to EGF membrane receptors. Competing activity is expressed as nanogram equivalents of EGF. Abbreviations: ST, media from ST-FeSV-transformed FRE; 5:2i and BHKi, media from cadmium and zinc-treated 5:2 and BHK cells, respectively; 5:2, media from 5:2 cells. (B) Two-milliliter equivalents of conditioned medium from cells described above were fractionated on a Bio-Sil TSK-250 high-pressure liquid chromatography column (21.5 by 600 cm) equilibrated in 40% acetonitrile-0.1% TFA, (pH 1.9). Fractions collected were lyophilized and tested for EGF-competing activity. Arrows denote eluting positions of the marker proteins: 68K, bovine serum albumin; 14K, RNase A; 6K, insulin.

FIG. 5. Pulse-chase analysis of the TGFα precursor protein. The 5:2 cells were induced for 10 h with cadmium and zinc, incubated in cysteine-free media for 1 h, and pulse-labeled with 1 mCi of $^{35}$S-cysteine per ml for 15 min. After pulse-labeling, cells were incubated with complete Dulbecco modified Eagle medium for various times, the TGFα precursor was immunoprecipitated, and immunoprecipitates were fractionated on a 15% polyacrylamide gel. Molecular weight markers (kilodaltons) are included on the left-hand side of the figure. Only the bottom two-thirds of the gel is shown.

FIG. 6. (A) Microsomal fractionation of the expressed TGFα precursor protein. The BHK and 5:2 cells were treated with cadmium and zinc for 8 h and labeled with $^{35}$S-cysteine for 12 h. The P100 and S100 fractions prepared as described in Materials and Methods were immunoprecipitated with anti-TGFα 137-151 and analyzed by gel electrophoresis on 12 to 20% gradient gels. +PEP indicates samples which were preincubated with synthetic peptide before being subjected to immunoprecipitation analysis. (B) Discontinuous sucrose gradient analysis of the P100 fraction from panel A. The sucrose interphases indicated were collected and immunoprecipitated with anti-TGFα 137-151. +PEP denotes samples blocked with synthetic peptide immunogen prior to immunoprecipitation analysis. The location of protein markers is shown to the left in each panel. Only the bottom two-thirds of the gel is shown.
6A. Nearly all the immunoreactive TGFα precursor protein was found in the P100 fraction (P100; lanes 5:2i), suggesting a membrane association. To delineate this potential membrane association further, the P100 fraction was separated by discontinuous sucrose gradient centrifugation. In this analysis, the plasma membrane fraction collects at the 20/35% sucrose interphase, since it possesses the lowest density of the microsomal components. Results of the immunoprecipitation analysis of the gradient fractions are shown in Fig. 6B. Densitometric analysis of the autoradiograph reveals that 52% of the $M_t$, 13,000 to 17,000 immunoreactive TGFα precursor fractionsate within the 20/35% sucrose interphase. Marker enzyme analysis shows that 64% of the membrane-associated 5'-nucleotidase activity (29) is located within this sucrose fraction (data not shown). These results suggest colocalization of the TGFα precursor with the plasmalemma fraction.

**DISCUSSION**

In this study, we expressed a transfected cDNA encoding the complete rat TGFα precursor in nontransformed BHK fibroblasts to study the expression and processing of the TGFα molecule. The expressed TGFα precursor protein was identified and characterized by site-specific immunological reagents directed toward peptide epitopes within the carboxy end of the precursor polypeptide. A summary of our results follows. First, the TGFα precursor was readily identified in the transfected BHK fibroblasts. The most stable from of this protein behaved on SDS-polyacrylamide gels as a diffusely migrating protein with apparent $M_t$, 13,000 to 17,000. This value is consistent with the predicted molecular weight of the precursor molecule. Second, immunoreactive proteins which appear to comigrate with the TGFα precursor detected in the BHK transfecant were found in two cell lines, Leydig testicular tumor and ST-FeSV-transformed FRE cells, known to produce TGFα naturally, but were not found in nontransformed FRE or BHK cells. Third, the $M_t$, 13,000 to 17,000 precursor molecule expressed in the transfected BHK cells localized with the plasmalemma of fractionated cells; this result is consistent with a proposed membrane localization of the precursor molecule based on an analysis of the TGFα cDNA (11). Finally, these nontransformed BHK fibroblasts were demonstrated to be capable of proteolytically processing the TGFα precursor molecule to the mature $M_t$, 6,000 species.

Pulse-chase experiments allowed us to examine the processing of the TGFα precursor molecule in intact BHK transfectants and to identify processing intermediates. Our results demonstrate that the $M_t$, 13,000 to 17,000 TGFα precursor arises from two short-lived intermediates of $M_t$, 14,000 to 18,000 and $M_t$, 13,000 to 19,000. The $M_t$, 14,000 to 18,000 polypeptide is the initial translation product observed after a short pulse-label and is rapidly converted into the larger, $M_t$, 15,000 to 19,000, intermediate. This initial processing event, which results in the apparent increase in molecular weight observed on SDS-polyacrylamide gels, may be the result of covalent posttranslational modification such as phosphorylation, lipidation, or carbohydrate addition. A typical N-linked glycosylation site is present in the predicted sequence of the TGFα precursor (11). The $M_t$, 15,000 to 19,000 TGFα precursor intermediate is subsequently converted into the $M_t$, 13,000 to 17,000 polypeptide. This final precursor form detected by the antiprotein antibody comigrates with the TGFα precursor identified in the BHK transfectant labeled with $^{[35}S]$cysteine under normal conditions. The loss in molecular weight of approximately 2,000 from the larger TGFα precursor intermediate is probably due to proteolysis. Antibodies directed toward the carboxy-terminal 7 amino acids of the TGFα precursor (anti-TGFα153-159) were also able to identify similar processing intermediates (data not shown). Thus, this early stage of proteolytic processing must occur at the amino end of the precursor molecule. A loss in molecular weight of 2,000 would be consistent with cleavage of the 23-amino-acid hydrophobic leader peptide predicted from the sequence of TGFα cDNA (13).

Complete processing of the $M_t$, 13,000 to 17,000 TGFα precursor polypeptide into the released $M_t$, 6,000 form of TGFα was evident in the supernatants of the transfected BHK fibroblasts. Media conditioned by these cells contained TGFα activity (as measured by EGF competition) which, upon gel permeation chromatography, coeluted with the fully processed 50-amino-acid TGFα form. Proteolytic cleavage of TGFα from its precursor occurs at Ala-Val-Val and is most probably mediated by a protease having elastase-type specificity (2, 11). The ability of nontransformed BHK fibroblasts to completely process the TGFα precursor indicates that these cells do express this protease and suggests that processing is not mediated by a tumor-specific proteolytic enzyme. It is entirely possible, however, that the levels of this protease are elevated in the malignant state. In this regard, it is interesting that while ST-FeSV-transformed FRE cells secrete significantly higher levels of the mature growth factor, they contain significantly lower levels of the $M_t$, 13,000 to 17,000 species. This is also true of the Leydig tumor cells (data not shown), suggesting that the half-life of the precursor is considerably shorter in malignant cells than in nontransformed BHK cells.

Several size classes of TGFα have previously been observed in the supernatants and extracts from tumor cells (1, 27). Although the $M_t$, 6,000 form of TGFα has been well characterized (15), its relationship with the larger forms of TGFα precursor is not well documented. Linsley et al. (12) and Ignotz et al. (8) used antipeptide sera directed toward the carboxy terminus of the 50-amino-acid TGFα molecule to characterize the larger secreted forms. Several molecular weight classes of immunoreactive material were identified, the most conspicuous of which was a polypeptide of molecular weight approximately 20,000 (8, 12). Ignotz et al. extended this analysis further and revealed that after digestion with elastase, this larger precursor form of TGFα released an $M_t$, 6,000 polypeptide with properties of mature TGFα (8). The relationship of this $M_t$, 20,000 secreted (released) form with the precursor forms detected in this report remains to be established. Our present results indicate that nontransformed BHK fibroblasts release only the mature fully processed 50-amino-acid TGFα. This raises the possibility that tumor cells process the TGFα precursor differently from nontransformed cells, resulting in the release of larger TGFα precursor forms. The precursor-specific antibodies described here should prove useful in assessing the relationship of these larger secreted forms with the TGFα precursor, as well as in characterizing the high-molecular-weight forms of TGFα-like species previously found in the urine of patients with disseminated cancer (21, 25).

Although much is known concerning the biological properties of TGFα, the role of this growth factor in the development of the malignant state remains unclear. The production of TGFα by a variety of transformed cells (24; Twardzik and Ranchalis, in press), as well as its occurrence in the
urine of cancer patients (21, 25), implies that it plays an important role in cellular transformation. Recently, Rosenthal et al. (19) presented evidence that the expression of the TGFα gene in nontransformed Rat-1 cells results in the acquisition of the transformed phenotype via an autocrine mechanism. Although we have not yet examined in detail the phenotype of the TGFα-expressing BHK cells, the precursor-processing capability of these nontransformed fibroblasts represents the initial stage of an autocrine response (22). We are currently examining the phenotype of these cells.

ACKNOWLEDGMENTS

We are grateful to Richard Palmer and Greg Mundy for cells, to Peter Linsley and Hans Marquardt for helpful discussions and critical comments, and to Nancy Olfs and Craig English for preparation of the manuscript.

This work was partially supported by Public Health Service grant CA 43793 to D.C.L. from the National Institutes of Health.

ADDITIONAL PROOF

While this manuscript was in press, Bringham et al. (T. S. Bringham, P. B. Lindquist, and R. Derynck, Cell 48:429–440, 1987) presented similar evidence describing the processing of the TGFα precursor.

LITERATURE CITED